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FUNGAL AND PARASITIC INFECTIONS

Peripheral Merozoite Surface Proteins Are Targets of Naturally Acquired Immunity against Malaria in both India and Ghana

Asier Garcia-Senosiain,[a,b] Ikhlaq Hussain Kana,[a,b] Susheel Kumar Singh,[a,b] Bishwanath Kumar Chourasia,[a,b] Manoj Kumar Das,[c] Daniel Dodoo,[d] Subhash Singh,[e] Bright Adu,[d] Michael Theisen[a,b]

[a]Centre for Medical Parasitology at Department of International Health, Immunology and Microbiology, University of Copenhagen and Department of Infectious Diseases, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark

[b]Department for Congenital Disorders, Statens Serum Institut, Copenhagen, Denmark

[c]National Institute of Malaria Research, Field Unit, Ranchi (Jharkhand), India

[d]Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana, Legon, Accra, Ghana

[e]Indian Institute of Integrative Medicine, Canal Road, Jammu, India

Asier Garcia-Senosiain and Ikhlaq Hussain Kana contributed equally to this article. Author order was determined alphabetically.

ABSTRACT Development of a successful blood-stage vaccine against *Plasmodium falciparum* malaria remains a high priority. Immune-epidemiological studies are effective tools for the identification of antigenic targets of naturally acquired immunity (NAI) against malaria. However, differences in study design and methodology may compromise interstudy comparisons. Here, we assessed antibody responses against intact merozoites and a panel of 24 recombinant merozoite antigens in longitudinal cohort studies of Ghanaian ($n = 115$) and Indian ($n = 121$) populations using the same reagents and statistical methods. Anti-merozoite antibodies were associated with NAI in both the Indian (hazard ratio [HR] = 0.41, $P = 0.020$) and the Ghanaian (HR = 0.17, $P < 0.001$) participants. Of the 24 antigen-specific antibodies quantified, 12 and 8 were found to be protective in India and Ghana, respectively. Using least absolute shrinkage and selection operator (LASSO) regression, a powerful variable subselection technique, we identified subsets of four (MSP6, MSP3.7, MSPDBL2, and Pf12) and five (cMSP3^{3D7}, MSP3.3, MSPDBL1, GLURP-R2, and RALP-1) antigens that explained NAI better than the individual antibodies in India (HR = 0.18, $P < 0.001$) and Ghana (HR = 0.31, $P < 0.001$), respectively. IgG1 and/or IgG3 subclasses against five antigens from these subsets were associated with protection. Through this comparative study, maintaining uniformity of reagents and methodology, we demonstrate that NAI across diverse geographic regions may result from antibodies to multiple antigenic targets that constitute the peripheral merozoite surface protein complexes.

KEYWORDS malaria, immunity, merozoite

Plasmodium falciparum malaria remains a major public health problem particularly in Africa and India, where more than 95% of all malaria cases worldwide were reported in 2017 (1).

Naturally acquired immunity (NAI) mediated by immunoglobulin G (IgG) antibodies against malaria develops over a long period with repeated infections (2–5). Antigenic targets of NAI appear to be conserved across geographically diverse regions since passively transferred IgG from immune West African adults to Thai malaria patients cleared parasitemia and alleviated symptoms (6). However, the mechanisms involved in NAI and the identity of the targets of protective IgG have not been fully elucidated. Since merozoites are exposed to circulating antibodies, merozoite surface proteins (MSPs) are likely targets of NAI (7–9). While immune epidemiological studies have been

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Address correspondence to Michael Theisen, mth@ssi.dk.

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TABLE 1 Demographics of study participants and febrile malaria status during follow-up

Demographic parameter	Subgroup	No. (%) in India	Subgroup	No. (%) in Ghana
Total no.		121		115
Age group	≤10 yr	48 (39.7)	≤5 yr	56 (48.7)
	11–15 yr	21 (17.4)	≥6 yr	59 (51.3)
	≥16 yr	52 (43.0)		
Sex	Female	59 (48.8)		59 (51.3)
	Male	62 (51.2)		56 (48.7)
Bed net use	Yes	14 (11.6)		41 (35.7)
	No	107 (88.4)		74 (64.3)
Follow-up status	Protected	73 (60.3)		42 (36.5)
	Baseline status			
	Parasitemic	62		23
	Nonparasitemic	11		19
	Susceptible	48 (39.7)		73 (63.5)
	Baseline status			
	Parasitemic	16		6
	Nonparasitemic	32		67

effective in identifying potential targets of protective antibodies, such studies have also produced conflicting data, with antibodies against the same antigen being protective in some studies but not in others (reviewed in reference 10). Possible reasons for such discrepancies may include (i) study design, (ii) statistical methodology, (iii) endpoint definition, (iv) antibody subclass profiles, (v) malaria transmission intensity, and (vi) ethnicity.

Here, we analyzed the patterns of NAI against *P. falciparum* malaria in two geographically diverse cohorts, one from India and the other from Ghana, using the same methodology, assay setup, endpoint definition, and statistical models. IgG antibody responses against whole merozoites and a panel of 24 recombinant merozoite proteins produced in a *Lactococcus lactis* expression system (11) were quantified. We evaluated their protective effect against febrile malaria to identify targets of NAI that transcend different epidemiological regions.

RESULTS

Study design and demographics. While anti-merozoite immunity is well documented in African populations, it is less described in India. Here, we comparatively assessed samples and clinical data from two longitudinal cohort surveys (LCS) conducted in Ghana and India (12, 13). For both study sites, malaria transmission is perennial but peaks during and after the rainy season and was relatively higher in Ghana (the parasite prevalence during LCS was 14.45% in Ghana and 8.36% in India). The peak incidence of febrile malaria occurred in younger children, up to 6 years of age (68.5% of all cases) in Ghana, whereas in India, peak incidence was up to 10 years of age (54.2% of all cases). The mean age of Indian participants (27.5 ± 18.8) was significantly higher than those in Ghana (5.7 ± 2.9; $P < 0.001$). At baseline (BL), blood samples were drawn from 386 Indian and 669 Ghanaian individuals, who were subsequently monitored for malaria case detection for 13 and 9 months, respectively. This study presents data for 121 Indians (31.3% of those sampled at BL) and 115 Ghanaians (17.2% of those sampled at BL) who were considered definitely exposed based on microscopically detected *P. falciparum* infection at any point during the respective study periods (see Fig. S1 in the supplemental material). Of these, 48 (39.6%) and 73 (63.5%) in India and Ghana, respectively, encountered at least one febrile malaria episode during the follow-up, and were termed susceptible to febrile malaria (Table 1).

Relationship between IgG antibodies against merozoites, parasitemia, and febrile malaria. To minimize the impact of methodological differences, anti-merozoite IgG was measured by flow cytometry-based immunofluorescence assay (FC-IFA) in samples from both cohorts using a common batch of freshly purified merozoites. Opsonizing IgG was significantly higher in individuals with asymptomatic *P. falciparum* infection than in those without infection at BL in both the Indian ($P = 0.018$) and the

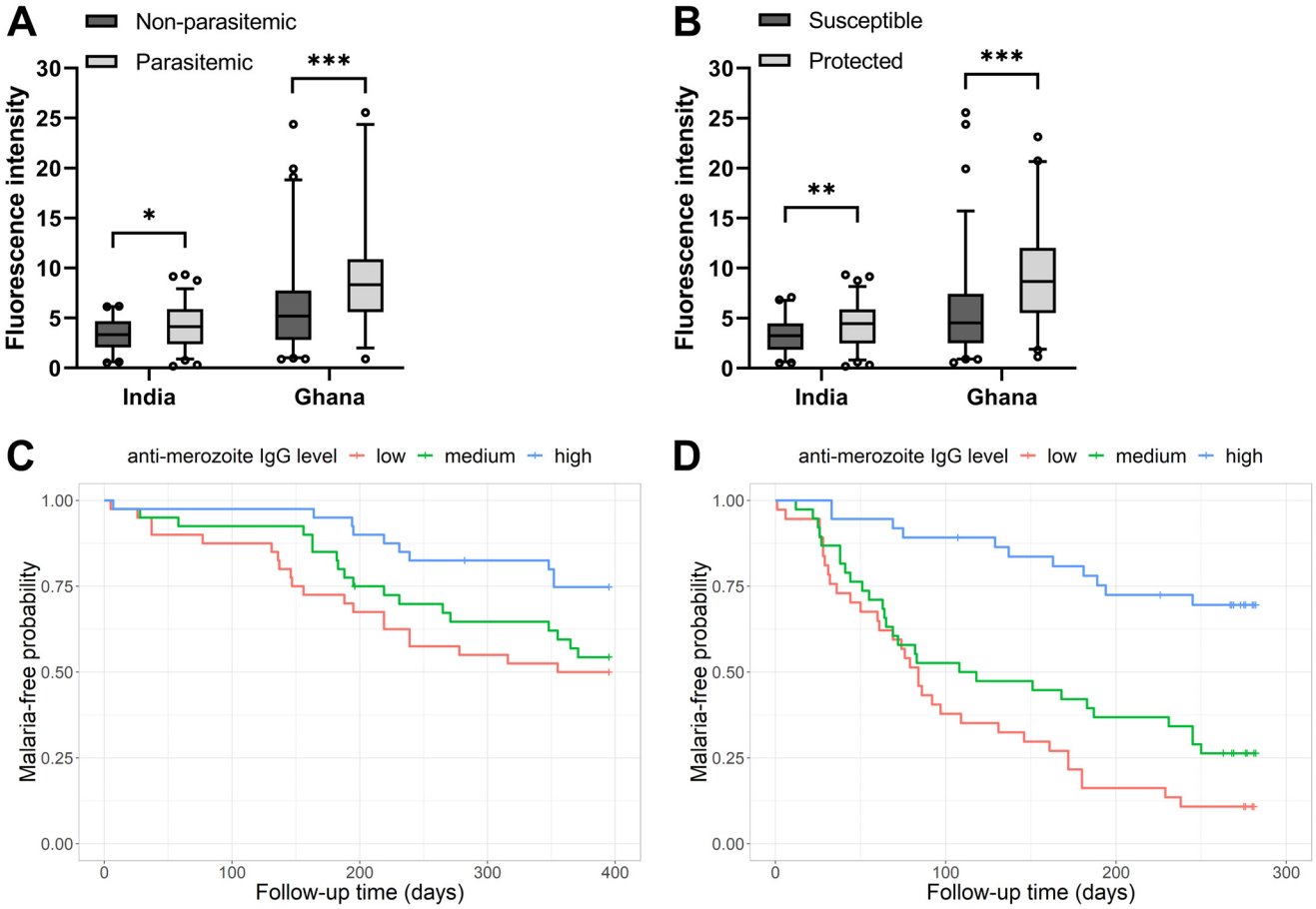

FIG 1 Antibodies against merozoites are associated with protection against febrile malaria. Fluorescence intensities were determined by FC-IFA. Antibody levels are shown stratified by infection at baseline (A) or by febrile malaria status during follow-up (B). The data are presented as box-and whisker plots showing the 5th to 95th percentiles. *P* values were determined by Mann-Whitney test. Asterisks represent *P* values (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (C and D) Antibody levels were stratified into three groups based on tertiles. Kaplan-Meier plots show the probability of remaining febrile malaria-free during the study period for individuals with low (red), medium (green), and high (blue) antibody levels against merozoites in India (C) and Ghana (D).

Ghanaian ($P < 0.001$) cohorts, suggesting a boosting effect of concurrent infections on antibody levels (Fig. 1A). The levels of antibodies were also higher in individuals who did not experience febrile malaria during follow-up (protected group) than in those who did (susceptible group) ($P = 0.008$ [India] and $P < 0.001$ [Ghana]) (Fig. 1B). Participants were categorized into low, medium, or high anti-merozoite IgG responders and assessed for the risk of febrile malaria in Cox regression models accounting for age. It was found that responders in the high IgG group had a significantly higher probability of remaining free of febrile malaria than those in the low IgG group in India (hazard ratio [HR] = 0.41; 95% confidence interval [CI] = 0.19 to 0.87; $P = 0.020$), as well as in Ghana (HR = 0.17; 95% CI = 0.08 to 0.34; $P < 0.001$; visualized as Kaplan-Meier plots in Fig. 1C and D). Collectively, these findings are consistent with the notion that anti-merozoite immunity develops globally and correlates positively with age and parasite exposure.

Association between antigen-specific antibodies and protection from febrile malaria. In an attempt to identify the molecular targets of NAI against merozoites, the levels of IgG antibodies against a panel of 24 recombinant merozoite antigens were measured by a multiplex assay (14) in both cohorts. Regarding the levels of merozoite IgG antibodies, both cohorts showed higher median levels of specific IgG in individuals who were (i) protected versus susceptible and (ii) parasitemic versus nonparasitemic at baseline (Fig. S2). Regardless of febrile malaria status and baseline parasitemia, the IgG antibody levels were generally higher in samples from Ghana than in those from India.

TABLE 2 Antigens associated with protection from febrile malaria in Indian and Ghanaian cohorts[a]

	India			Ghana		
Antigen	**HR (95% CI)**	***P***	**Adj *P***	**HR (95% CI)**	***P***	**Adj *P***
Surface GPI anchored						
$MSP1_{19K}$	0.21 (0.08–0.57)	**0.002**	0.052	0.81 (0.45–1.46)	0.488	1.000
$MSP2^{3D7}$	0.59 (0.26–1.33)	0.207	1.000	0.44 (0.23–0.84)	**0.013**	0.308
$MSP2^{FC27}$	0.45 (0.18–1.15)	0.094	1.000	0.45 (0.25–0.81)	**0.008**	0.196
Pf12	0.33 (0.13–0.79)	**0.013**	0.322	0.56 (0.30–1.04)	0.066	1.000
Pf38	0.46 (0.19–1.09)	0.078	1.000	0.75 (0.42–1.37)	0.354	1.000
Peripherally associated						
GLURP-R0	0.30 (0.12–0.76)	**0.012**	0.276	0.82 (0.45–1.51)	0.528	1.000
GLURP-R2	0.21 (0.08–0.55)	**0.002**	**0.037**	0.41 (0.21–0.80)	**0.009**	0.212
MSP3.3	0.30 (0.12–0.77)	**0.013**	0.300	0.86 (0.47–1.58)	0.621	1.000
MSP3.7	0.28 (0.11–0.71)	**0.007**	0.172	0.72 (0.38–1.35)	0.308	1.000
$cMSP3^{3D7}$	0.27 (0.10–0.73)	**0.010**	0.231	0.51 (0.28–0.95)	**0.035**	0.837
$nMSP3^{3D7}$	0.71 (0.29–1.71)	0.443	1.000	0.56 (0.28–1.13)	0.104	1.000
$nMSP3^{K1}$	0.53 (0.22–1.26)	0.151	1.000	0.68 (0.36–1.27)	0.224	1.000
MSP6	0.30 (0.12–0.77)	**0.013**	0.305	0.57 (0.31–1.04)	0.068	1.000
MSPDBL1	0.81 (0.33–2.02)	0.656	1.000	0.43 (0.22–0.85)	**0.015**	0.361
MSPDBL1 (Leucine)	0.49 (0.20–1.18)	0.110	1.000	0.74 (0.42–1.31)	0.304	1.000
MSPDBL2	0.43 (0.18–1.02)	0.056	1.000	0.42 (0.21–0.83)	**0.012**	0.291
SERA5	0.63 (0.24–1.60)	0.329	1.000	1.06 (0.58–1.92)	0.856	1.000
Rhoptry						
PfRh2a	0.26 (0.10–0.71)	**0.008**	0.201	0.51 (0.28–0.93)	**0.028**	0.675
PfRh2b	0.39 (0.16–0.97)	**0.043**	1.000	0.68 (0.35–1.33)	0.259	1.000
PfRh2-2030	0.59 (0.22–1.58)	0.294	1.000	0.81 (0.43–1.54)	0.516	1.000
RALP-1	0.41 (0.16–1.08)	0.070	1.000	0.82 (0.45–1.49)	0.513	1.000
RAMA	0.23 (0.08–0.68)	**0.008**	0.185	0.54 (0.28–1.07)	0.079	1.000
RON4	0.47 (0.18–1.24)	0.127	1.000	0.31 (0.15–0.64)	**0.001**	**0.036**
Microneme						
EBA140RIII-V	0.34 (0.13–0.87)	**0.025**	0.607	0.72 (0.40–1.29)	0.266	1.000

[a]IgG levels against a panel of merozoite antigens were analyzed for their protective effect against febrile malaria. A Cox proportional-hazard model comparing high and low responders (divided into tertiles) was used to determine the hazard ratios (HR), 95% confidence intervals (95% CI), unadjusted *P* values (*P*), and Bonferroni adjusted *P* values (Adj *P*) for each antibody response. Age group and merozoite IFA were included in the model as confounders for exposure. Antigens are grouped according to their subcellular localization. Significant *P* values (<0.05) are indicated in boldface.

The probability of developing febrile malaria during follow-up for each antibody level (categorized as low, medium, or high) was assessed by Cox regression analyses adjusted for age and the FC-IFA readouts for anti-merozoite IgG. Using the low IgG category as a reference for the respective antigens, we found that high IgG levels to 12 and 8 antigens were inversely related to the probability of developing febrile malaria in India and Ghana, respectively (Table 2). Of these, IgG responses to GLURP-R2, PfRh2a, and $cMSP3^{3D7}$ were common to both cohorts. After applying the Bonferroni correction for multiple testing, only one association remained significant in each cohort: GLURP-R2 in India and RON4 in Ghana.

Breadth of antibodies and protection from febrile malaria. There is evidence that protection from febrile malaria most likely results from multiple antibody specificities (15–17). To evaluate associations between breadth of antigen-specific IgG and malaria immunity, we generated a breadth score (a linear variable) for each participant based on the magnitude of all 24 antibody specificities. As expected, the breadth score increased with age in both India and Ghana ($P < 0.001$; Fig. 2A and B). When individuals were stratified by febrile malaria status during follow-up, the group of protected individuals displayed higher breadth scores than susceptible individuals ($P < 0.001$; Fig. 2C and D). The breadth score was also significantly higher in individuals who were parasitemic at BL than nonparasitemic individuals both in India ($P = 0.004$) and in Ghana ($P < 0.001$).

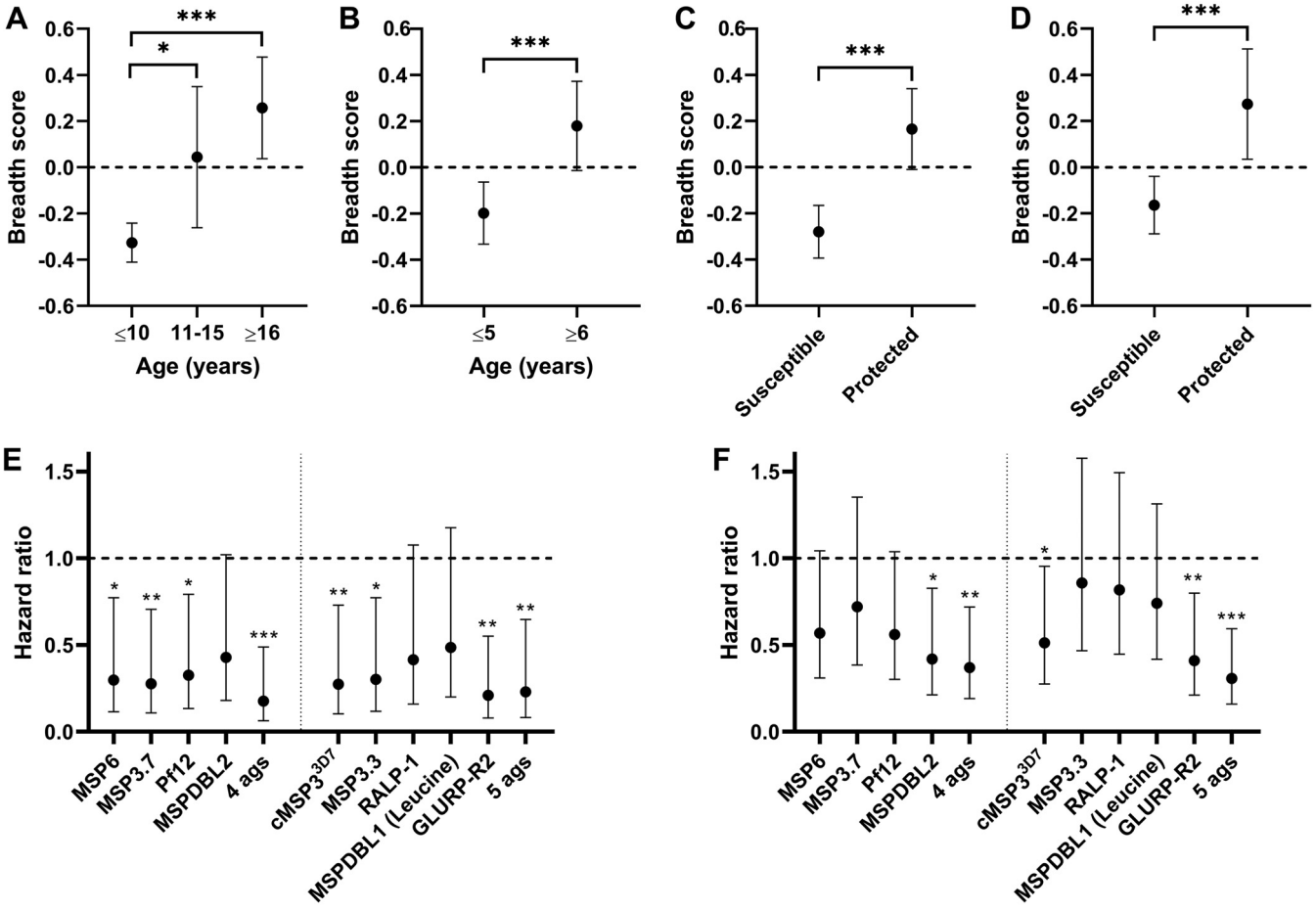

FIG 2 Breadth of antibody responses in relation to age and febrile malaria. Mean breadth scores in India (A and C) and Ghana (B and D) stratified by age (A and B) or febrile malaria status during follow-up (C and D). Error bars represent 95% confidence intervals. *P* values were determined by Kruskal-Wallis test or Mann-Whitney test. The Cox proportional-hazard model comparing high and low responders was used to calculate hazard ratios, 95% confidence intervals, and *P* values for each antibody response or combination in India (E) and Ghana (F). The model was adjusted for age and antibody levels against whole merozoites. The horizontal dashed line indicates no association with protection (HR = 1). The vertical dotted line divides the subsets of antigens and combinations identified in India and Ghana (left and right, respectively). Asterisks represent *P* values (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Next, we used the least absolute shrinkage and selection operator (LASSO) regression and 10-fold cross-validation analysis method (18) to select combinations of antibody specificities that could explain protective immunity better than any individual response. By using this approach, IgG responses against antigen subsets identified for the Indian and Ghanaian cohorts consist of those against four (MSP6, MSP3.7, MSPDBL2, and Pf12) and five (cMSP3^{3D7}, MSP3.3, MSPDBL1 [Leucine], GLURP-R2, and RALP-1) antigens, respectively. Cox proportional-hazard models used to determine estimates of protection confirmed that these combinations of four and five antibody specificities were strongly associated with protection in India (HR = 0.18, $P < 0.001$) and Ghana (HR = 0.31, $P < 0.001$), respectively (Fig. 2E and F). The protective associations of antibody reactivities against the combination of antigens identified in each cohort were stronger than the combination of all antigens (HR = 0.30 and 0.61 in India and Ghana, respectively) or any of the individual antibody reactivities detected in the respective cohort. Importantly, the antigen combination identified in the Ghanaian cohort was also a strong predictor of protection from febrile malaria in the Indian cohort (HR = 0.23) and vice versa (HR = 0.37).

Profiling IgG subclasses against each antigen subset in the respective populations. Antibodies of the cytophilic subclasses (19) and in particular IgG3 (14, 20–23) are associated with immunity against blood-stage *P. falciparum*. It was therefore of interest to determine the subclass profile of naturally acquired IgG against these two

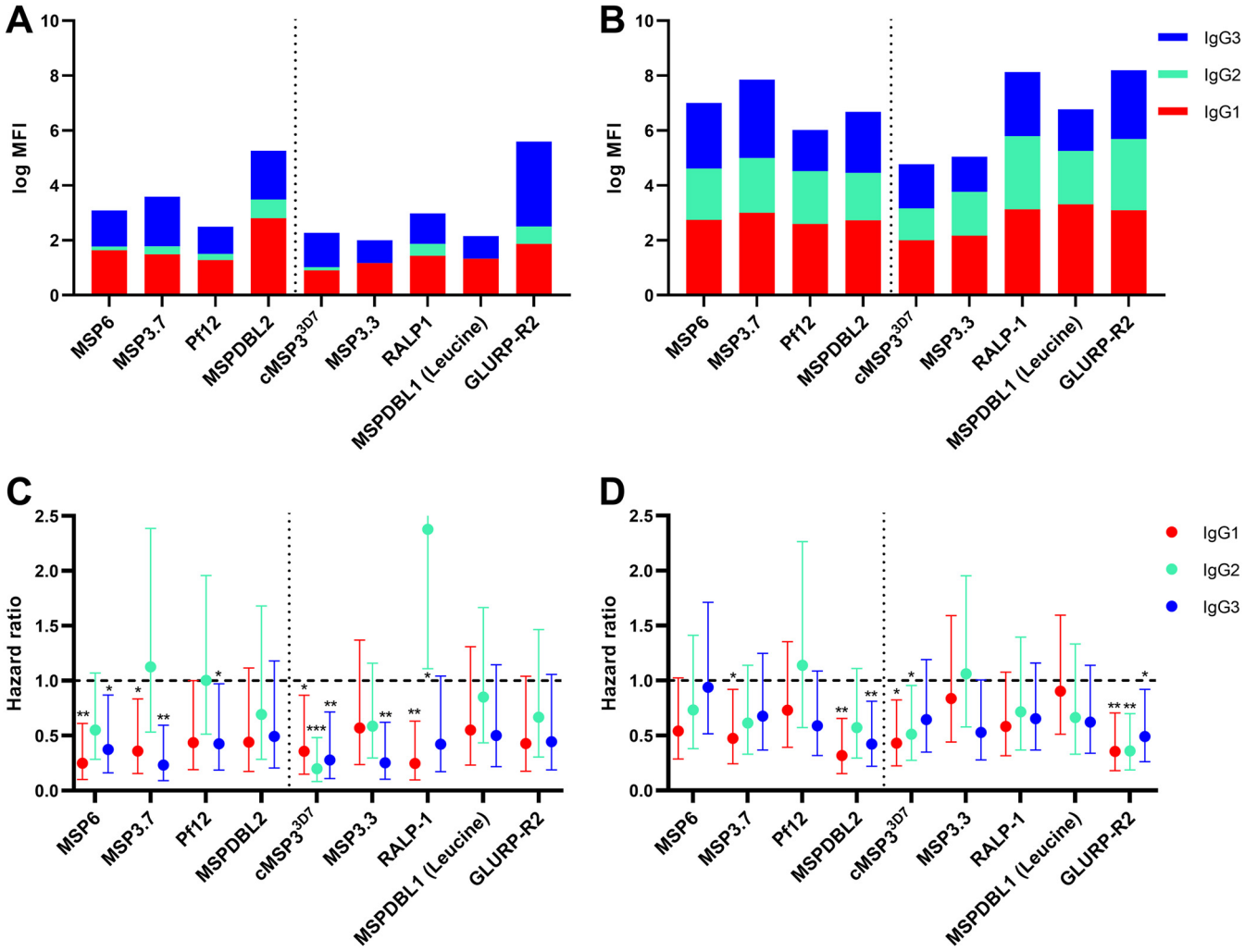

FIG 3 IgG subclass responses against selected antigens and their association with protection. Bars represent the median of $\log_{10}$-transformed fluorescence intensities (MFIs) values of immunoglobulin G (IgG) subclass (IgG1, IgG2, and IgG3) in samples from India (A) and Ghana (B), respectively. The vertical dotted line divides the subsets of antigens identified in India (left) and Ghana (right). (C and D) Associations of IgG subclass responses with protection from febrile malaria in Indian and Ghanaian cohorts, respectively. Hazard ratios, 95% confidence intervals, and *P* values were calculated using a Cox proportional-hazard model comparing high and low responders. The models were adjusted for age and antibody levels against whole merozoites. The horizontal dashed line indicates no association with protection (HR = 1). Asterisks represent *P* values (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

antigen subsets. It appears that there is a predominance of cytophilic antibodies against both antigen subsets in both cohorts (Fig. 3A and B). Specific IgG subclasses were categorized as low, medium, or high based on the respective mean fluorescence intensities (MFIs). For the antigen subset identified in India, high levels of IgG subclasses against MSP6 ($HR_{IgG1} = 0.25$ and $HR_{IgG3} = 0.37$), MSP3.7 ($HR_{IgG1} = 0.36$ and $HR_{IgG3} = 0.23$), and Pf12 ($HR_{IgG3} = 0.43$) in the Indian cohort (Fig. 3C) and MSP3.7 ($HR_{IgG1} = 0.47$) and MSPDBL2 ($HR_{IgG1} = 0.32$ and $HR_{IgG3} = 0.42$) in the Ghanaian cohort (Fig. 3D) were significantly associated with a higher probability of remaining malaria-free during the follow-up period. For the antigen subset identified in Ghana, high levels of IgG subclasses against GLURP-R2 ($HR_{IgG1} = 0.36$, $HR_{IgG2} = 0.36$, and $HR_{IgG3} = 0.49$) and $cMSP3^{3D7}$ ($HR_{IgG1} = 0.43$ and $HR_{IgG2} = 0.51$) in the Ghanaian cohort (Fig. 3D) and against $cMSP3^{3D7}$ ($HR_{IgG1} = 0.36$, $HR_{IgG2} = 0.20$, $HR_{IgG3} = 0.28$), MSP3.3 ($HR_{IgG3} = 0.25$), and RALP-1 ($HR_{IgG1} = 0.25$) in the Indian cohort (Fig. 3C) were significantly associated with protection against febrile malaria.

DISCUSSION

Identification of molecular targets of NAI has been hampered not only by an incomplete understanding of the underlying mechanisms but also by conflicting

evidence from several immune epidemiological studies involving the use of different isoforms of same antigens, variations in the detection of antibody reactivities, and differences adopted in data analysis. Using harmonious experimental methodology and identical data analysis plan, we have demonstrated that antibodies against whole merozoite preparation, and certain combinations of recombinant antigens (from a panel of 24) in particular, are involved in NAI against malaria in geographically diverse populations from Africa and India.

First, using a common batch of purified merozoites, we found that high levels of merozoite IgG were associated with a reduced probability of febrile malaria in both cohorts from India and Ghana. Second, we assessed specific IgG reactivities toward a panel of 24 recombinant merozoite antigens using common reagents. These recombinant antigens were subdomains from different merozoite proteins selected from different subcellular compartments, most of which have been identified to play mechanistic roles in erythrocyte invasion and have been identified as targets of protective immune responses in various studies (14, 15, 24–26). In both study cohorts, antibody reactivity to whole merozoites, as well as to the majority of the recombinant antigens, individually and in combination, increased with age and was higher in participants who were parasitemic at baseline, thus reinforcing the notion that an active infection boosts preexisting malarial antibodies. In general, the levels of specific IgG antibodies were higher in the Ghanaian than in the Indian cohort, most likely due to higher malaria transmission in Ghana. Next, we prospectively assessed antibody associations with protective immunity and found that antibodies against 12 and 8 antigens were individually associated with protection against febrile malaria in India and Ghana, respectively. To identify those antibody specificities which are most strongly associated with protection from febrile malaria, we used the LASSO regression and 10-fold cross-validation analysis method, which aids in selecting subsets of relevant predictors and can be applied to Cox proportional-hazard models. LASSO has been successfully applied to create predictive models in multiple human diseases (27–31). By using this approach, we identified two antigen subsets—(i) MSP6, MSP3.7, MSPDBL2, and Pf12 and (ii) cMSP3^{3D7}, MSP3.3, MSPDBL1 (Leucine), GLURP-R2, and RALP-1—that explained protective immunity better than any individual antibody reactivity in the Indian and Ghanaian cohorts, respectively. Our data thus reinforce previous findings that the cumulative responses to combinations of antigens are a better predictor of protection than responses to their single-antigen components (25, 32), and these combinations may vary among populations. It is not fully understood why different subsets of antigens were more protective in the two cohorts; however, further planned studies on both parasite genetic variability and host major histocompatibility complex (MHC) class II restrictions in the cohorts may help explain some of these observations.

From the Indian subset of antigens, IgG3 subclass antibody levels against Pf12, MSP3.7, and MSP6 were also associated with protection, whereas in a previous study in the same cohort, MSP6-specific IgG3 antibodies were found to be nonprotective (14). This is due to variation in the data analysis plan. Previously, protective association of each antigen was assessed by multivariate logistic regression analysis (fitting IgG3 data as a linear covariate) (14), while in the present study IgG3 data (categorized into tertiles) were assessed by Cox proportional-hazard models. In an earlier study, two of the antigens (GLURP-R2 and cMSP3^{3D7}) from this Ghanaian subset were also found to correlate with protective immunity and opsonic phagocytosis of merozoite activity when these Ghanaian IgG preparations were assessed in an antigen-coated bead-based phagocytosis assay (26).

Taken together, these observations suggest that multiple antibody specificities are involved in NAI and that the exact combination of such antibodies differs from one region to another, possibly due to differences in population-specific MHC class II restrictions and circulating parasite strains (antigenic polymorphism). Interestingly, seven of the antigens identified as targets of NAI in our study, both in the Indian and the Ghanaian cohorts, are peripherally associated MSPs. Of these, six belong to the

MSP3 family of proteins (MSP6, MSP3.7, and MSPDBL2 from the Indian cohort and cMSP3^{3D7}, MSP3.3, and MSPDBL1 [Leucine] from the Ghanaian cohort), and one is GLURP-R2, which was recently characterized as another merozoite surface protein (5). A network of cross-reactivity has been reported among the conserved domains of MSP3 family of proteins, where antibodies elicited against any member of this family (depending on the host MHC class II restrictions) could exert an antiparasite effect through reactivity against other members of the family (33). In this study, we used unique domains from members of MSP3-family of proteins, which do not share appreciable sequence similarities. However, it is intriguing that study participants could have developed antibody responses toward other domains of the MSP3 family of proteins, including those against the conserved cross-reactive domains. Indeed, in the present study we detected IgG responses against cMSP3^{3D7} in both the Indian and the Ghanaian cohorts, which were found to be associated with protection against febrile malaria. IgG responses to conserved cross-reactive domains of other MSP3 family of proteins remains to be tested. Curiously, one rhoptry-neck protein Pf12 and RALP-1 were identified in the Indian and Ghanaian cohorts, respectively. While Pf12 is a GPI-anchored protein (34), RALP-1 is associated through coiled-coil domains with an unknown protein and colocalizes with RON4, another rhoptry-neck protein (35). Whereas RON4 was the strongest predictor of protection in the Ghanaian cohort, this was not the case in the Indian cohort.

Most of the NAI targets identified in the present study have been previously described as targets of functional antibodies with antiparasite effects, either through neutralization of the merozoite invasion in erythrocytes or in cooperation with mononuclear cells through antibody-dependent cellular inhibition and opsonic phagocytosis (5, 26, 36–39). It should be noted that our experimental setup has the inherent limitation of not completely reflecting the *in vivo* situation since we quantified antibody levels with a multiplex assay, using recombinant proteins, rather than assessing antigen-specific antibodies in functional assays, which are likely to be better correlates of protective immunity. Though IgM antibodies have been relatively understudied, they have been found to inhibit merozoite invasion of erythrocytes in a complement-dependent manner. Along with IgG, their levels have also been significantly associated with protective immunity against malaria and therefore warrant further investigation to validate their role in malaria immunity (40).

In conclusion, to the best of our knowledge, this is the first report about the identification of targets of NAI using comparative immune epidemiological studies in geographically diverse cohorts from Africa and India. Findings from this study reinforce the observations from earlier studies that anti-merozoite IgG is important in NAI against febrile malaria; notwithstanding, the methodological synchrony in such immune epidemiological studies, involving either whole parasites or individual proteins, is critical for consistent results. Furthermore, using a well-suited statistical tool for the identification of antigenic targets, we found that cumulative antibody responses to two distinct combinations of peripheral merozoite surface antigens were strongly associated with protective immunity in both Indian and Ghanaian study populations.

MATERIALS AND METHODS

Ethics statement. The studies were approved by the Institutional Review Board of Noguchi Memorial Institute for Medical Research of the University of Ghana, Accra, Ghana, and by the Institutional Ethics Committee of the National Institute of Malaria Research, Indian Council of Medical Research, New Delhi, India. Written informed consent was given by the study participants or their guardians before enrollment in the study.

Study areas, populations, and design. The Indian study was conducted in Dumargarhi in the state of Jharkhand and has been described in detail previously (13). A total of 945 individuals (ages 1 to 82 years) were enrolled in the study, which lasted from May 2014 to September 2016. It included four cross-sectional surveys in which subsets of the population were sampled, and a 13-month longitudinal cohort survey (LCS) between April 2015 and April 2016. During this period, trained field workers visited the village every fortnight for active surveillance, which included recording the temperature for all febrile individuals.

The African study was conducted in Asutsuare, Damgbe, West District, in Ghana, as previously described in detail (12). In total, 798 children under 12 years were enrolled in May 2008. Venous blood was obtained at enrollment and children were followed up actively and passively for malaria detection in a 42-week LCS.

At both sites, febrile malaria was defined as any *P. falciparum* parasitemia confirmed by microscopy of stained thick and thin blood smears plus reported fever or axillary temperature ≥37.5°C at the time of the visit. Individuals who suffered at least one case of febrile malaria during follow-up were considered susceptible, while those who did not experience any episodes of febrile malaria despite having parasites at BL were considered protected.

Parasite culture and merozoite isolation. *P. falciparum* strain NF54 was cultured in O^+ human erythrocytes at a parasitemia between 1 and 5% with a 3% hematocrit. Parasite growth medium consisted of RPMI 1640 with 25 mM HEPES, 2 mM L-glutamine, 25 μg/ml gentamicin, 0.5% AlbuMAX, and 2% heat-inactivated serum. Parasites were kept at 37°C in an atmosphere containing 5% O_2, 5% CO_2, and 90% N_2. Parasitemia and developmental stage were monitored by preparing thin blood smears. Smears were fixed with methanol, stained with 10% Giemsa for 10 min, and observed by light microscopy. Parasites were synchronized by treatment with 5% sorbitol for 10 min. Early schizonts were harvested with a magnetic separation column and then cultured in parasite growth medium. Mature schizonts were filtered through a 1.2-μm-pore filter, and hemozoin was removed by passing through an LS MACS column three times.

Flow cytometry-based immunofluorescence assay. Flow cytometry-based immunofluorescence assay (FC-IFA) was performed as previously described (14). In brief, purified merozoites (4×10^5 per well) were added to a 96-well U-bottom plate. Test samples were added, followed by incubation for 1 h with shaking. Plates were washed twice with wash buffer (0.5% bovine serum albumin in phosphate-buffered saline [PBS]). Then, 100 μl of phycoerythrin-conjugated sheep anti-human IgG antibodies diluted 1:1,000 was added. After incubation for 1 h and two washes, the merozoites were resuspended in 200 μl of wash buffer. Plates were read in a Beckman Coulter cytometer (5,000 events). The results were analyzed using Kaluza analysis software.

Multiplex assay for antibody measurement. A panel of 24 *P. falciparum* recombinant antigens (41) was covalently coupled to internally labeled microspheres according to the manufacturer's instructions (Luminex). Antibody quantification has been described in detail previously (41). In brief, a mix containing approximately 1,250 beads of each of the antigen-coupled bead regions was added to each well of a prewetted 96-well filter microtiter plate. The plates were then washed three times with assay buffer E (ABE; 0.1% bovine serum albumin, 0.05% Tween 20, and 0.05% sodium azide in PBS [pH 7.4]). Test samples were added at 100 μl per well and incubated for 2 h with shaking. After three washes with ABE, 100 μl of phycoerythrin-labeled goat anti-human IgG antibodies (Jackson Immuno Research) was added at a dilution of 1:3,500. The plates were then incubated for 1 h with shaking and washed three times with ABE. After three washes and resuspension of the samples in 100 μl of ABE, the plates were read in a Luminex 200 (Bio-Rad Laboratories, Inc.).

Data analysis. The Mann-Whitney test was used to evaluate differences between two groups. For comparisons between more than two groups, the Kruskal-Wallis test and Dunn's multiple-comparison test were used. *P* values of less than 0.05 were considered significant. The Cox proportional-hazard model was used to determine the association of antibody levels and breadth scores with protection. The levels and scores were classified into three groups (low, medium, and high) based on tertiles with models comparing the low and high groups. Age and anti-merozoite IgG levels were included in the models as confounders. Age was stratified into three levels in India (≤10, 11 to 15, and ≥16 years) and into two levels in Ghana (≤5 and ≥6 years), as previously reported (5, 14, 39, 42). In antigen-specific total IgG, subclasses or breadth score association analyses, we adjusted for age and anti-merozoite IgG levels as confounders because both of these variables are (i) a proxy for parasite exposure, (ii) correlated with malaria disease outcome, and (iii) correlated with each other. In the past, we (14, 26) and others (32) have used a similar approach in antibody association studies. As published previously, bed net usage and gender were not identified as confounders (5, 14).

To generate the breadth scores, antigen-specific antibody levels were first normalized for each cohort separately using the following formula:

$$z = \frac{x - \bar{x}}{\sigma},$$

where x is the observation, $\bar{x}$ is the mean, and σ is the standard deviation of the samples. Each individual's z values for all 24 antigens were averaged to calculate the breadth score.

The LASSO regression model was applied to select antibody combinations strongly associated with protection. Normalized antigen-specific IgG levels were included in the model. Variables with little predictive value were reduced to zero, which reduced the number of variables, resulting in a simpler final model (Fig. S3A and B). The appropriate lambda value was selected using 10-fold cross-validation and optimizing for low deviance (Fig. S3C and D). New breadth scores were calculated as described previously for the antibody combinations selected with the LASSO procedure.

Statistical analysis was performed using Prism 8 (GraphPad Software, Inc.) and R version 3.5.1 (43). The packages survival (44, 45) and glmnet (46) were used for modeling and cross-validation.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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S.S. is a consultant at Virdis BioPharma Pvt., Ltd., Mumbai, India.

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